Free Radical Research, Vol. 35, pp. 779–788
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Anticarcinogenic Antioxidants As Inhibitors Against Intracellular Oxidative Stress

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Accepted by Professor E. Niki

(Received 5 March 2001; In revised form 27 April 2001)

Oxidative stress has been implicated in the pathogenesis of numerous diseases, including cancer. In the present study, the protective effect of natural antioxidants, such as quercetin and tea polyphenols, on intracellular oxidative stress was studied. Here we report a novel function of quercetin and tea polyphenols, as potential inhibitors of 4-hydroxy-2-nonenal (HNE)-induced intracellular oxidative stress and cytotoxicity. In rat liver epithelial RL34 cells, a potent electrophile HNE dramatically induced the productions of reactive oxygen species (ROS), which correlated well with the reduction in cell viability. We found that quercetin and tea polyphenols, such as epigallocatechin gallate and theaflavins and their gallate esters, significantly inhibited the HNE-induced ROS production and cytotoxicity. In addition, HNE induced a transient decrease in the mitochondrial membrane potential ($\Delta\psi$), which was also retarded by the antioxidants. These data suggest that the antioxidants, such as quercetin and tea polyphenols, are inhibitors against mitochondrial ROS production.

Keywords: 4-hydroxy-2-nonenal; Oxidative stress; Antioxidant; Quercetin; Tea polyphenols; Mitochondria

Abbreviations: EGCG, epigallocatechin gallate; HNE, 4-hydroxy-2-nonenal; ROS, reactive oxygen species; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DiOC6(3), 3,3′-dihexyloxacarbocyanine iodide; GSH, glutathione; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

INTRODUCTION

Oxidative stress is increasingly seen as a major upstream component in the signaling cascade involved in many of the cellular functions, such as cell proliferation, inflammatory responses, stimulating adhesion molecule, and chemoattractant production.[1] It has been suggested that some level of oxidative stress may be required in response to cytotoxic agents and converted into the redox regulatory system as a downstream signaling pathway.[2] However, excess oxidative stress may be toxic, exerting cytostatic effects,

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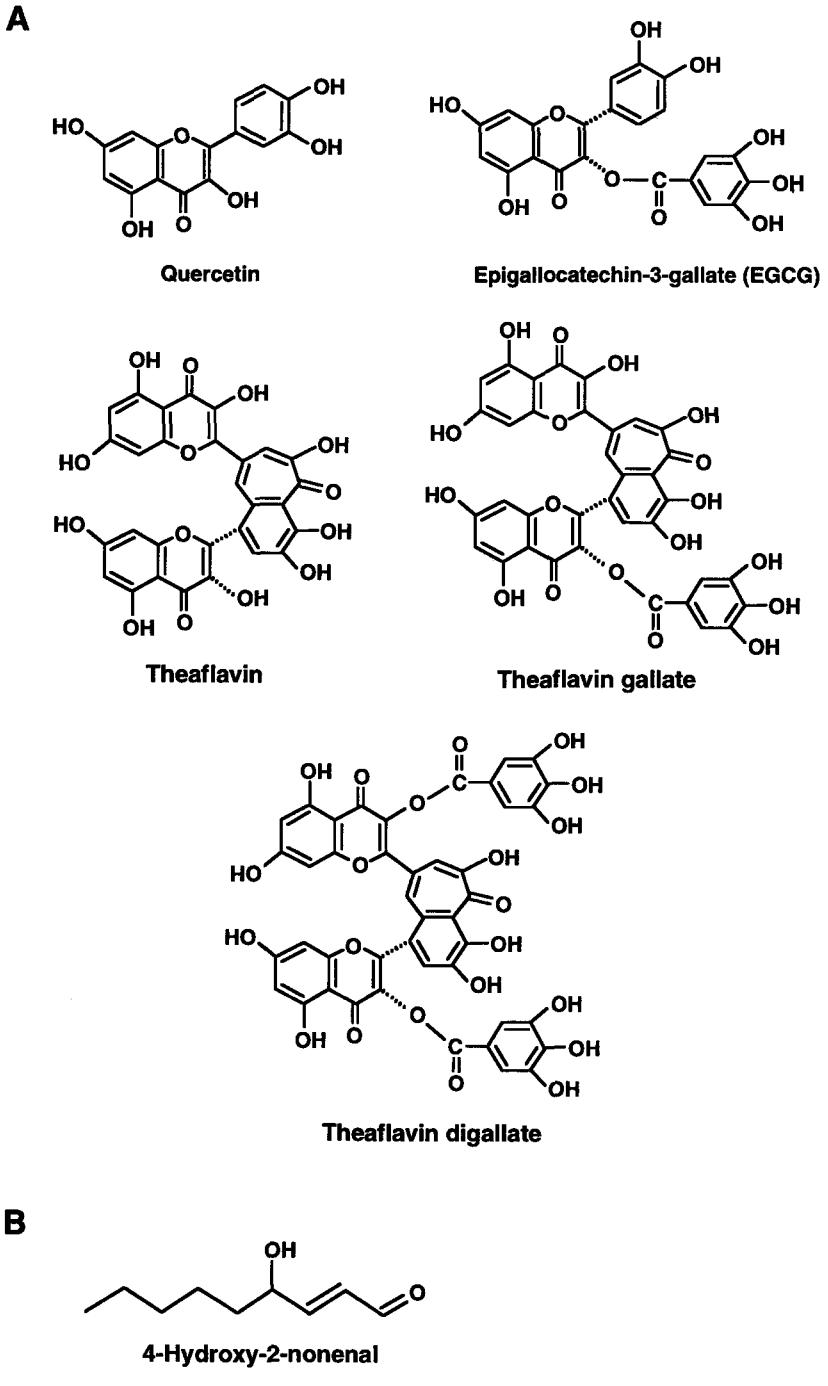

FIGURE 1 Chemical structures of quercetin and tea polyphenols (A) and HNE (B).

causing membrane damage, and activating pathways of cell death (apoptosis and/or necrosis). Reactive oxygen species (ROS) generated during oxidative stress may be responsible for these effects due to their ability to damage cellular components, such as membrane lipids. Lipid peroxidation mediated by a free radical chain reaction mechanism yields lipid hydroperoxides as primary products, and subsequent decomposition of the lipid hydroperoxides generates a large number of reactive aldehydes, such as ketoaldehydes, 2-alkenals, and 4-hydroxy-2-alkenals.[3,4] Therefore, these aldehydes represent the end products of oxidative stress; however, they are still highly reactive to various biomolecules, such as proteins and DNA, and may contribute to carcinogenesis.[5–10]

Many phenolic compounds from plants are considered to have protective effects for cells and organisms. Quercetin (Fig. 1A), the representative flavonoid that would be ubiquitously found in foods of plant origin, is reported to have a number of biological functions in cell. For example, quercetin possesses antioxidant properties including the prevention of the oxidation of low density lipoproteins *in vitro*,[11] the protection of colonocyte DNA from H_2O_2 attack,[12] inhibition of H_2O_2-induced NF-κB DNA binding activity,[13] and has the ability to inhibit chemically induced tumorigenesis in rodents.[14,15] Whereas, as a large group of the polyphenolic compounds, tea polyphenols have also been found to be beneficial to human health. A number of intensive studies have mainly been done on the green tea polyphenols, such as epigallocatechin gallate (EGCG), and black tea polyphenols, such as theaflavin, theaflavin gallate, and theaflavin digallate (Fig. 1A). It has been shown that these polyphenols have antioxidative, anticarcinogenic, and antiviral activities in diverse biological systems.[16–20] The exact mechanisms responsible for the anticarcinogenic effect of these natural antioxidants, however, is not yet thoroughly understood.

We have recently found that 4-hydroxy-2-nonenal (HNE) (Fig. 1B), a potent mutagenic and genotoxic electrophile endogenously produced during lipid peroxidation, is a potential inducer of intracellular oxidative stress.[21] This raised the possibility that the biological effect of HNE is closely associated with the production of ROS in the cells. In the present study, we have studied the effect of several anticarcinogenic antioxidants, such as quercetin and tea polyphenols, on the HNE-induced ROS production and cytotoxicity. Our studies demonstrated for the first time that these antioxidants could function as inhibitors against intracellular oxidative stress, which may represent a major causative factor on the electrophile-induced cytotoxicity.

MATERIALS AND METHODS

Materials

HNE was obtained from Cayman Chemicals. Quercetin was obtained from Wako Chemical (Osaka, Japan). The purified tea polyphenols, such as EGCG, theaflavin, theaflavin gallate, and theaflavin digallate, were kindly provided by Mitsui Norm, (Japan).

Cell Culture

RL34 cells were obtained from the Japanese Cancer Research Resources Bank. The cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), L-glutamine (588 μg ml^{-1}), and 0.16% $NaHCO_3$ at 37°C in an atmosphere of 95% air and 5% CO_2. Cells post-confluency were exposed to the lipid peroxidation products in DMEM containing 5% FBS.

Cytotoxicily

Cells suspended in the medium with FBS were plated at 10^4 cells well^{-1} in 96-well plates. After a 24 h incubation at 37°C, the cells were exposed to HNE in the medium. After incubation, the cells were treated with 10 μl of MTT solution (5 mg ml^{-1}) for 4 h and then treated with 100 μl of 0.04N HCl–isopropanol solution. Cell viability was determined by formazan production from diphenyltetrazolium salt using a multiplate reader at 570 nm (630 reference filter).

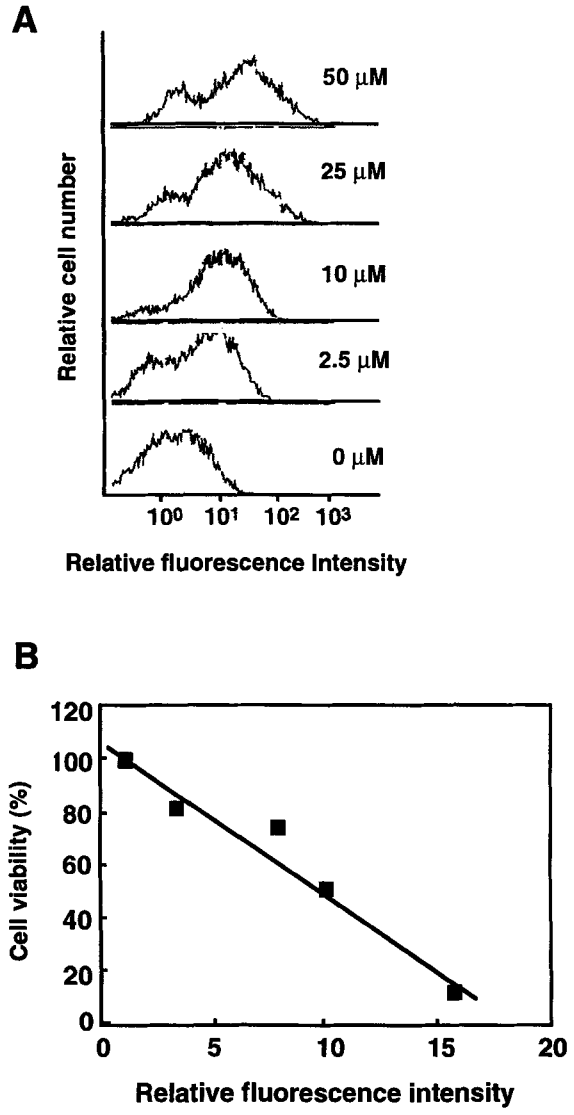

FIGURE 2 HNE-induced production of ROS and cytotoxicity. (A) ROS production in the cells exposed to HNE. The cells incubated with DCFH-DA (10 μM) for 30 min and then exposed to 25 μM HNE for 1 h. The fluorescence intensity of more than 10,000 cells was analyzed using a flow cytometer. (B) Correlation between cytotoxicity and ROS production in the cells exposed to HNE. Cells were exposed to HNE (0–50 μM), and ROS production and cell viability were measured.

Detection of Intracellular ROS Production and Mitochondrial Membrane Potential ($\Delta\psi$)

Cells were incubated with 5 μM 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) (dissolved in dimethylsulfoxide) for 30 min at 37°C and then treated with different agents for an additional 30 min at 37°C. After chilling on ice, the cells were washed with ice-cold PBS, scraped from the plate, and resuspended at 1×10^6 cells ml^{-1} in PBS containing 10 mM EDTA. For the detection of $\Delta\psi$, 40 nM 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3))[22] in the absence or presence of HNE was added and incubated for 15 min at 37°C. The fluorescence was measured using a flow cytometer (Epics XL, Beckman Coulter).

RESULTS

Correlation Between Intracellular Oxidative Stress and Cytotoxicity

On the basis of the previous finding that HNE is a potent inducer of intracellular ROS production in hepatocytes,[21] we have investigated whether the production of ROS within the cells is associated with the reduction of cell viability. The intracellular ROS level was determined using DCFH-DA as the intracellular fluorescence probe. As shown in Fig. 2A, the level of ROS induced by HNE was increased in a dose-dependent manner. The ROS level at 25 μM HNE was approximately 10-fold higher than that of the control. In addition, we found that the HNE-induced ROS

production was correlated well with the HNE cytotoxicity (Fig. 2B). These data suggest that HNE showed its cytotoxicity via the intracellular production of ROS.

Effect of Phenolic Antioxidants on Intracellular Oxidative Stress

Epidemiological studies have suggested that natural antioxidants, such as flavonoids and tea polyphenols, could protect against human cancer.[23–26] A number of animal studies have also attested to their anticarcinogenic activity.[27–33] Hence, we investigated the cytoprotective potential of quercetin and tea polyphenols, such as EGCG and theaflavins and their gallate esters (Fig. 1), focusing on the inhibition of HNE-induced cytotoxicity and intracellular ROS production. As shown in Fig. 3A, the treatment of cells with quercetin led to a significant inhibition of HNE cytotoxicity. In addition, the inhibition of cytotoxicity by quercetin correlated with the inhibition of intracellular ROS production (Fig. 3B). The tea polyphenols, such as EGCG, theaflavin, theaflavin gallate, and theaflavin digallate, also showed similar effects (Fig. 4), and their activities were apparently related to the structure of the compounds. The hierarchy of activity of these polyphenols as inhibitors of HNE cytotoxicity and ROS production is: EGCG = theaflavin digallate > theaflavin gallate > theaflavin. These antioxidants also showed a significant protective effect against cytotoxicity induced by *tert*-butylhydroperoxide or the glucose–glucose oxidase superoxide-generating system (data not shown), whereas antioxidative vitamins, such as α-tocopherol and ascorbate, were nearly ineffective (data not shown).

Source of ROS Production and Effect of Antioxidants

We have recently found that the pretreatment of diethyl maleate, which blocks the intracellular thiol groups, including mitochondrial GSH, produced intracellular peroxides and enhanced the electrophile-stimulated oxidative stress.[34] Moreover, the pretreatment by diphenylene iodonium, acting not only as a NAD(P)H oxidase inhibitor but also as an inhibitor of mitochondrial ROS production, resulted in a significant decrease in the electrophile-stimulated accumulation of ROS.[34] These observations suggest that the oxidative stress detected in the cells exposed to HNE may originate from the mitochondria, one of the major ROS-producing organella. In this context, we measured the alteration of the mitochondrial membrane potential ($\Delta\psi$), which is a component of the overall proton motive force that drives the ATP production in the mitochondria after exposure to HNE, and examined the effect of antioxidant treatment. As shown in Fig. 5A, HNE induced a significant decrease in the mitochondrial $\Delta\psi$, which correlated to the enhanced production of ROS (Fig. 2), suggesting that the ROS detected in the cells exposed to HNE may originate, at least in part, from the mitochondria. We then examined the effect of antioxidants on the HNE-induced alteration of $\Delta\psi$. As shown in Fig. 5B, the treatment of quercetin led to a significant inhibition of the alterations of $\Delta\psi$. Tea polyphenols also showed similar inhibitory effects against the alteration of $\Delta\psi$ (data not shown), which was correlated with their inhibitory potential against cytotoxicity and ROS production.

DISCUSSION

It has been demonstrated that chemically mediated ROS play important roles in mutagenesis and carcinogenesis.[35] Particular attention has been drawn to the relation between oxidative stress and tumor promotion because many tumor promotors, such as TPA, were shown to activate phagocytic and nonphagocytic cells to generate ROS. These strengthen the evidence that ROS are involved in tumor promotion, and

that generation of ROS and the subsequent oxidative DNA modification are related to the tumor-promoting potencies of various types of tumor promotors.[36] In the previous study, we found that the lipid peroxidation product, HNE, is a potential source of intracellular ROS.[21] This finding suggested that HNE could indirectly cause cellular damage via the production of ROS. Although the detailed mechanism for the HNE-induced ROS production is currently unknown, the accumulating data suggest the involvement of mitochondria. Indeed, HNE induced a

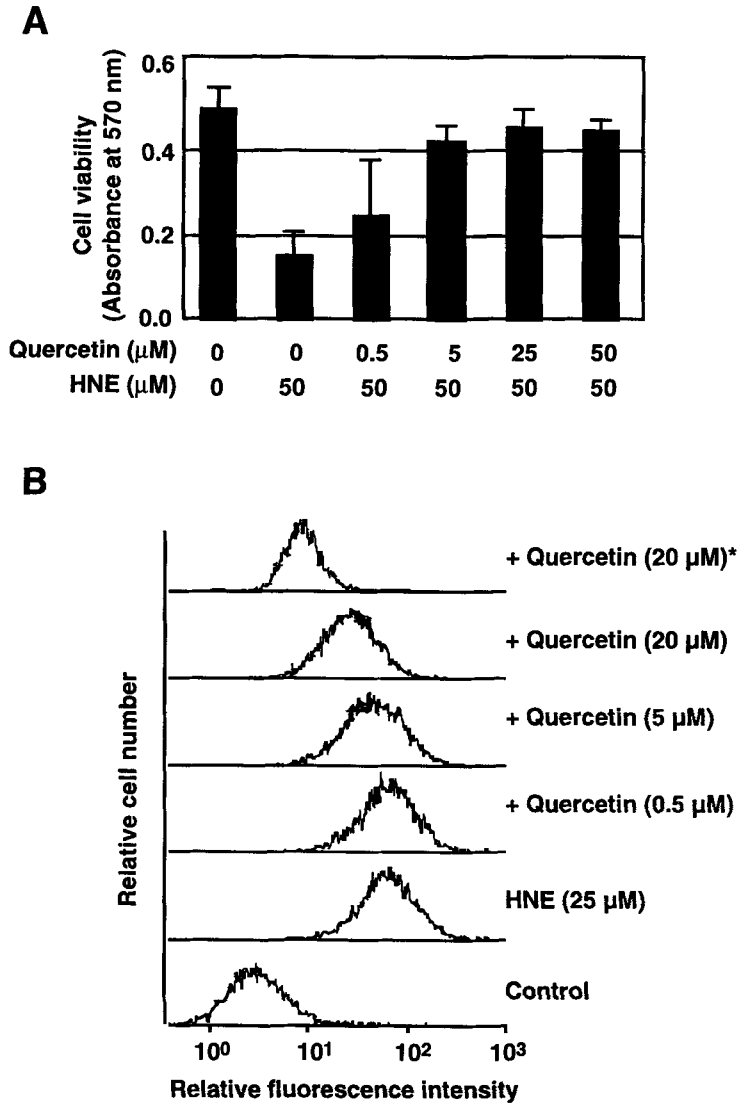

FIGURE 3 Effect of quercetin on HNE-induced intracellular oxidative stress. (A) Effect of quercetin on HNE cytotoxicity. The cells pretreated with quercetin (0–50 μM) for 4 h were exposed to 50 μM HNE and the cell viability was measured by the MTT assay. (B) Effect of quercetin on ROS production induced by HNE. The cells pretreated with quercetin (0–20 μM) for 4 h were incubated with 10 μM DCFH-DA for 30 min and then treated with 25 μM HNE for 1 h. After washing with PBS, the cells were resuspended in PBS containing 10 μM EDTA and then the fluorescence intensity of more than 10,000 cells was analyzed using a flow cytometer. *Simultaneous treatment of cells with HNE and quercetin.

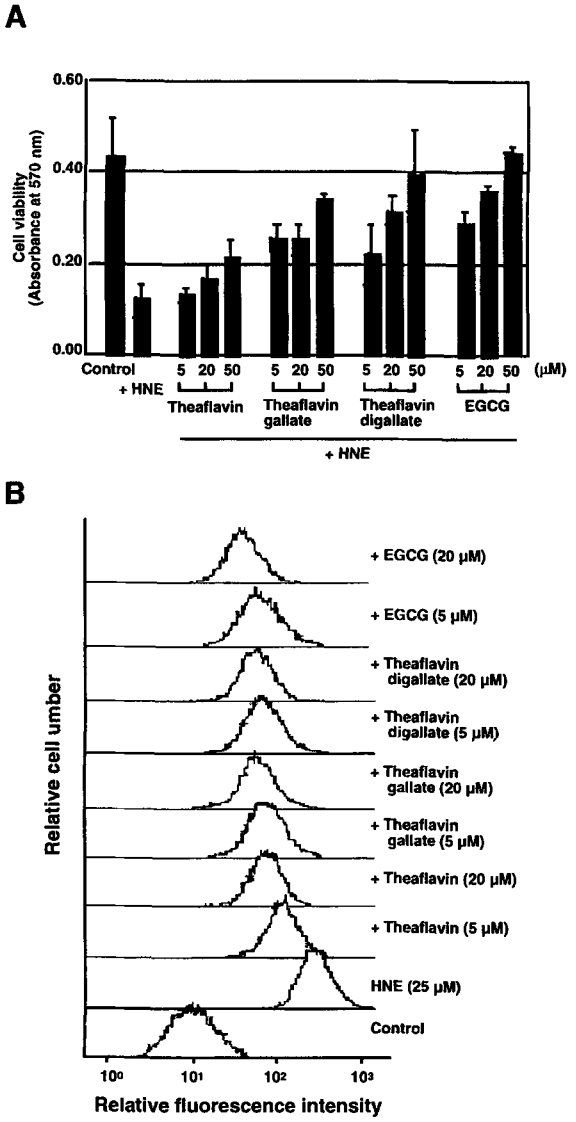

FIGURE 4 Effect of tea polyphenols on HNE-induced intracellular oxidative stress. (A) Effect of tea polyphenols on HNE cytotoxicity. The cells pretreated with tea polyphenols (5, 20, or 50 μM) for 4 h were exposed to 50 μM HNE and the cell viability was measured by the MIT assay. (B) Effect of tea polyphenols on ROS production induced by HNE. The cells pretreated with tea polyphenols (5 or 20 μM) for 4 h were incubated with 10 μM DCFH-DA for 30 min and then exposed to 25 μM HNE for 1 h. After washing with PBS, the cells were resuspended in PBS containing 10 μM EDTA and then the fluorescence intensity of more than 10,000 cells was analyzed using a flow cytometer. *Simultaneous treatment of cells with HNE and quercetin.

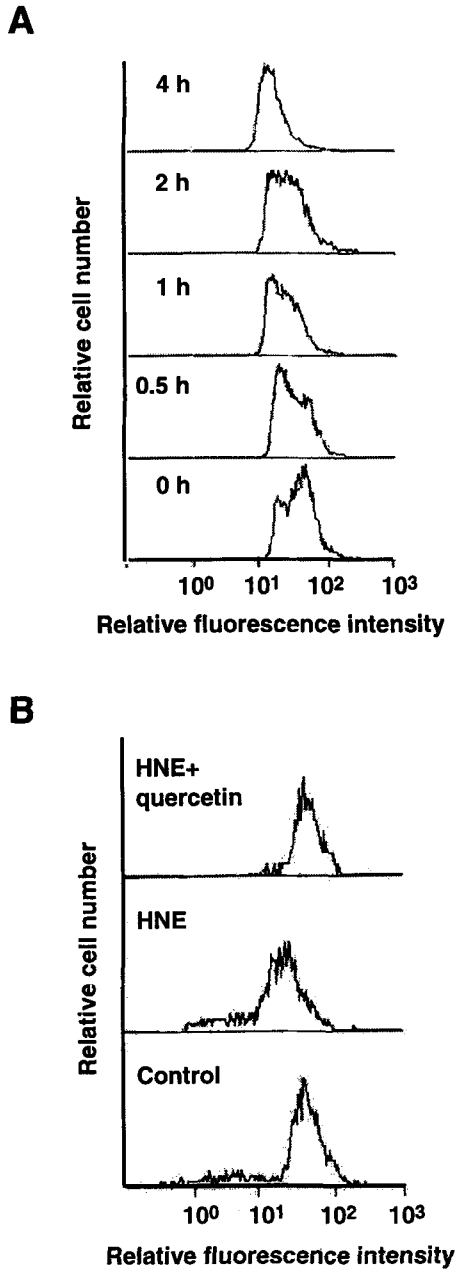

FIGURE 5 Time-dependent alterations of mitochondrial membrane potential ($\Delta\psi$) included by HNE (A) and effect of quercetin (B).

significant decrease in the mitochondrial $\Delta\psi$ (Fig. 5), which agreed with the enhanced production of ROS in the HNE-treated cells. When $\Delta\psi$ decreases, the breakdown of the outer membrane of mitochondria may occur.[37–39] During this process, ROS and even HNE by itself could react with the thiol groups of GSH localized at the mitochondrial membrane level[40] and contribute to a lower GSH level. A low level of GSH could favor the decrease in $\Delta\psi$, which would subsequently activate the opening of permeability transition pores to finally induce the release of the apoptosis-inducing factor[37] and/or of cytochrome c.[41] Alternatively, HNE could directly or indirectly modify the mitochondrial membrane proteins, leading to the alteration of $\Delta\psi$. Indeed, the HNE-modified mitochondrial proteins have been detected in trophoblast cells of preeclamptic placentas.[42]

A number of studies have demonstrated the antioxdiative properties of flavonoids and tea polyphenols. Quercetin has been shown directly to scavenge ROS, such as the superoxide anion and hydroxyl radical, based on the action of hydroxyl groups in the aromatic B ring.[43–46] It has also been established that the radical scavenging action of catechins requires the presence of the catechol moiety in the B ring.[47] EGCG, among the catechins, exhibits the highest antioxidative activity in various *in vitro* systems, and the presence of three hydroxyl groups side by side in the B ring and galloyl moiety has been suggested to be important for this action.[47] The structure-activity relationship study of theaflavins has been demonstrated that the antioxidative and antimutagenic activities are dependent on the number of galloyl moieties.[20] The present study also revealed that the protective potency of theaflavins against the HNE-induced cytotoxicity correlated with their structures (Fig. 4). Their cytoprotective effects were consistently potentiated with the number and arrangement of the phenolic hydroxy groups, suggesting that these polyphenols may act, in part, as free radical scavengers in the cells.[48]

It has been demonstrated that in certain cells, the activation of mitogen-activated protein kinases (MAPKs) in response to growth factors is associated with the production of ROS.[2] A number of studies have shown that the phenolic compounds activate MAPKs as the pro-oxidants.[49,50] We have recently reported that HNE significantly activates MAPKs, such as c-Jun N-terminal kinase and p38, and that the HNE-induced MAPK activation is markedly inhibited by quercetin.[21] In addition, it has also been shown that tea polyphenols, such as EGCG, significantly inhibit the activity of MAPKs in human epidermal carcinoma cells.[32] These observations suggest that one potential target for the intracellular oxidative stress is MAPKs and the removal of ROS by the antioxidants may interrupt the signal transductions associated with cell proliferation and tumor progression. Beyond that, both flavonoids and polyphenols have a wide spectrum of biological activities that may contribute to cancer chemopreventive effects. It has been demonstrated that quercetin inhibits the growth of malignant cells through various mechanisms: inhibition of glycolysis, macromolecule synthesis and enzymes, freezing cell cycle, and interaction with estrogen type II binding sites.[51] In addition, quercetin has been reported to inhibit the induction of heat shock proteins and thermotolerance without affecting other protein synthesis.[52] Whereas, tea polyphenols have also been shown to modulate the enzymes associated with cellular proliferation and carcinogenesis.[16] The results in the present study suggest the possibility that one of the molecular mechanisms of anticarcinogenesis by flavonoids and tea polyphenols is mediated by inhibition of the intracellular ROS production, leading to proliferative signal blocking and differentiative signal modulation in the target cells. Therefore, modulation of the redox state of the cell may provide one mechanism to explain the observation that some antioxidants appear to exert protective effects against human cancers.

Acknowledgements

We are grateful to Dr Yukihihiko Hara (Mitsui Norin Co. Ltd) for supplying tea polyphenols. This work was supported in part by Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

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